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Anti-Inflammatory Agents

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| <b>13. ABSTRACT (Maximum 200 Words)</b><br><p>Following studies with ibuprofen that demonstrated radiation sensitization with prostate cancer cells in vivo and in vitro, we are examining the potential targets of cellular radiosensitization. We observed that NSAIDs inhibited the prostaglandin synthesis at much lower concentrations than those required to induce cytotoxicity and radiosensitization, suggesting that the cellular sensitization appears to be a target other than cyclooxygenases. The mechanisms currently being explored include inhibition of NF-kB. Apoptotic pathways controlled by the Rel/NF-kB family of transcription factors may regulate the response of cells to DNA damage. In the cell lines tested, NF-kB was constitutively activated in hormone-independent PC3 and DU145 cells but was activated only by cytokine or radiation in hormone-sensitive LNCaP cells. Ibuprofen inhibited constitutive as well as stimulated NF-kB activity. We studied the activity of IKK-<math>\alpha</math> kinase, which mediates NF-kB activation. In PC3 cells the kinase activity was constitutively active, whereas LNCaP cells had minimal kinase activity that was activated by TNF-<math>\alpha</math>. Ibuprofen inhibited the constitutive activation of IKK-<math>\alpha</math> kinase in PC3 cells and blocked stimulated activation of IKK-<math>\alpha</math> kinase in LNCaP cells. The constitutive activation of NF-kB in prostate cancer cells may increase expression of anti-apoptotic proteins, thereby decreasing the effectiveness of anti-tumor therapy.</p> |   |  |  |                               |
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## TABLE OF CONTENTS

|                              |   |
|------------------------------|---|
| Cover                        | 1 |
| SF 298                       | 2 |
| Foreword                     | 3 |
| Table of Contents            | 4 |
| Research Accomplishments     | 5 |
| Key Research Accomplishments | 6 |
| Conclusions                  | 6 |
| References                   | 7 |
| Appendices                   | 8 |

### **Research accomplishments (October, 1998-September,1999)**

During the period of this report some of the experiments outlined in Task 1 (role of Nf-kB) and Task 2 (cyclooxygenases), Statement of work, have been undertaken.

Ibuprofen, a nonsteroidal anti-inflammatory agent (NSAID), enhances the effects of radiation on prostate cancer cells *in vitro* as well as *in vivo* (1,2). Although recent literature indicates that NSAIDs may have diverse cellular effects (3,4,5), the primary mechanism of action of NSAIDs has been considered to be inhibition of prostaglandin synthesis by inhibition of cyclooxygenases COX-1 and COX-2 (6 ). We investigated the effects of NSAIDs on prostaglandin synthesis in PC3 human prostate cancer cells (Task 2, Statement of work). Several NSAIDs tested inhibited arachidonic acid-induced prostaglandin synthesis at micromolar concentrations (Figure 1, Appendix). However, both the cytotoxic and radiosensitizing effects of NSAIDs required much higher concentrations than those required to inhibit the prostaglandin synthesis, suggesting that other molecular mechanisms may be responsible for the cytotoxicity and radiosensitization. Nonetheless, studies on COX-1 and COX-2 will be continued as these enzymes appear to be involved in angiogenesis(7) and thus may be of significant importance in *in vivo*.

The transcription factor NF-kB is reportedly activated by radiation and other types of DNA damage and activation is associated with suppression of apoptosis and increased cell survival. Inhibition of NF-kB appears to increase the sensitivity to cytotoxic agents and radiation (8). To determine if the observed ability of ibuprofen to enhance the cytotoxicity of radiation occurs through the inhibition of NF-kB, we examined the role of NF-kB in prostate cancer cells (Task 1, Statement of work). We found that NF-kB was constitutively activated in hormone-independent PC3 cells and there was no further significant increase in NF-kB following treatment with radiation or TNF- $\alpha$ . Another hormone-independent cell line DU145 also had constitutively activated NF-kB. In contrast, in hormone-sensitive LNCaP cells, NF-kB DNA-binding activity was seen only upon stimulation with TNF- $\alpha$  or radiation. Ibuprofen inhibited the constitutive as well as the stimulated NF-kB activity. We also measured the activity of IKK- $\alpha$  kinase, which mediates NF-kB activation by phosphorylation of the inhibitory protein I $\kappa$ B- $\alpha$  (9). In PC3 cells IKK- $\alpha$  was constitutively activated and there was no further significant stimulation by TNF- $\alpha$ . In LNCaP cells IKK- $\alpha$  activity was stimulated by TNF- $\alpha$ . Ibuprofen inhibited the constitutive and stimulated activation of IKK- $\alpha$  kinase. However, ibuprofen did not directly inhibit the kinase suggesting ibuprofen inhibits an upstream regulator of IKK- $\alpha$  kinase. These results are now

published (Palayoor et al, Constitutive activation of I $\kappa$ B kinase  $\alpha$  and NF- $\kappa$ B in prostate cancer cells is inhibited by ibuprofen. *Oncogene*, 18, 7389-7394, (1999). (Reprint, Appendix)

**Key Research accomplishments:**

Our results are the first demonstration that NF- $\kappa$ B is constitutively activated in hormone-independent prostate tumor cell lines, but not in an androgen-sensitive cell line.

**Reportable outcomes:**

**Publication:**

S.T. Palayoor, M.Y. Youmell, S.K. Calderwood, C.N. Coleman and B.D. Price, Constitutive activation of I $\kappa$ B kinase  $\alpha$  and NF- $\kappa$ B in prostate cancer cells is inhibited by ibuprofen. *Oncogene*, 18, 7389-7394, 1999.

**Conclusions:** NF- $\kappa$ B is constitutively activated in hormone-independent prostate cancer cell lines PC3 and DU145 but not in hormone-responsive LNCaP cells. The constitutive activation of NF- $\kappa$ B may increase expression of anti-apoptotic proteins, thereby decreasing the effectiveness of anti-tumor therapy. NSAIDs may prove to be valuable drugs for use in treatment of tumors, and may provide starting point for the rational design of agents which specifically inhibit the activation of NF- $\kappa$ B.

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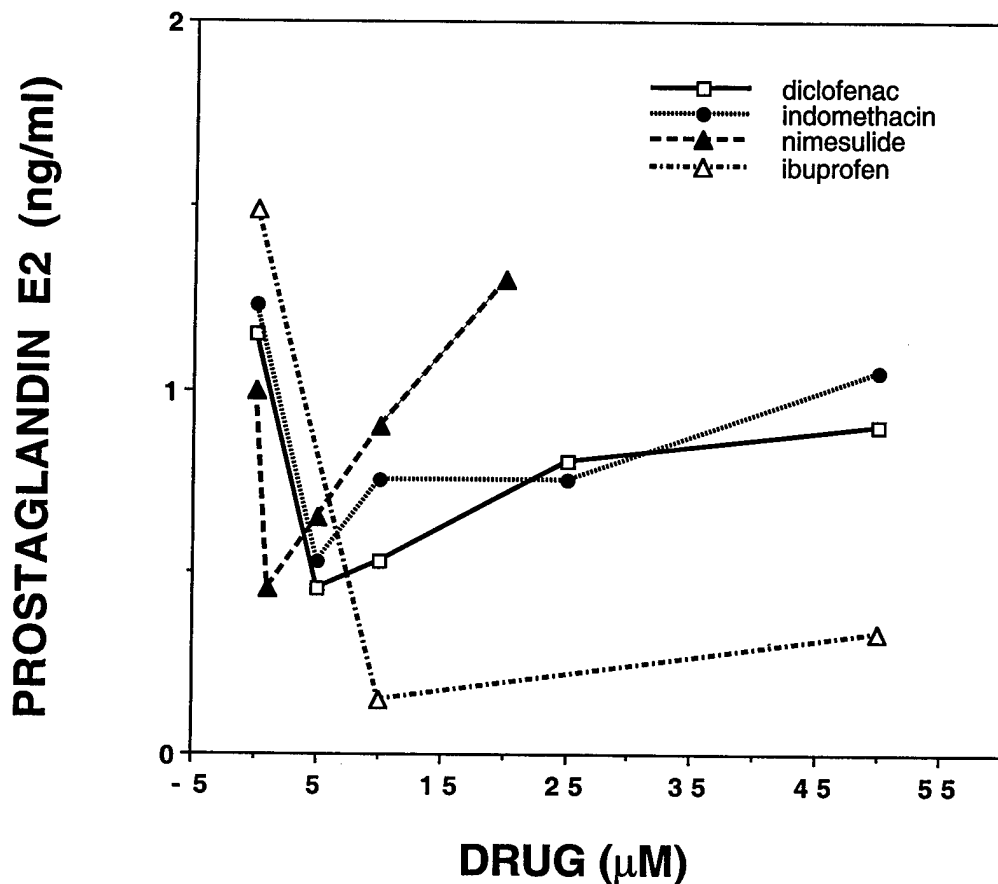
## **Appendices**

- 1) Figure 1: NSAIDs inhibit prostaglandin synthesis at micromolar concentrations.
- 2) Reprint: S.T. Palayoor, M.Y. Youmell, S.K. Calderwood, C.N. Coleman and B.D. Price, Constitutive activation of I $\kappa$ B kinase  $\alpha$  and NF- $\kappa$ B in prostate cancer cells is inhibited by ibuprofen. *Oncogene*, 18, 7389-7394, 1999.



FIGURE 1

## NSAIDs INHIBIT PROSTAGLANDIN SYNTHESIS AT MICROMOLAR CONCENTRATIONS



The effect of ibuprofen and other NSAIDs on arachidonic acid (AA)-induced prostaglandin synthesis was determined by ELISA (Oxford Biomedical Res, Inc.) assay. PC3 cells were plated in 6-well plates and treated for 10min with NSAIDs in the presence or absence of 30 μM AA. Media was collected and analyzed for prostaglandin E2. The basal PGE2 levels were  $0.36 \pm 0.07$  ng/ml (n=8). 30 μM AA increased PGE2 to  $1.33 \pm 0.18$  ng/ml. NSAIDs inhibited PGE2 synthesis at micromolar concentrations. Nimesulide is a COX-2 inhibitor.



## SHORT REPORT

**Constitutive activation of I $\kappa$ B kinase  $\alpha$  and NF- $\kappa$ B in prostate cancer cells is inhibited by ibuprofen**ST Palayoor<sup>2</sup>, MY Youmell<sup>1</sup>, SK Calderwood<sup>1</sup>, CN Coleman<sup>2</sup> and BD Price<sup>\*1</sup>

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Apoptotic pathways controlled by the Rel/NF- $\kappa$ B family of transcription factors may regulate the response of cells to DNA damage. Here, we have examined the NF- $\kappa$ B status of several prostate tumor cell lines. In the androgen-independent prostate tumor cells PC-3 and DU-145, the DNA-binding activity of NF- $\kappa$ B was constitutively activated and I $\kappa$ B- $\alpha$  levels were decreased. In contrast, the androgen-sensitive prostate tumor cell line LNCaP had low levels of NF- $\kappa$ B which were upregulated following exposure to cytokines or DNA damage. The activity of the I $\kappa$ B- $\alpha$  kinase, IKK $\alpha$ , which mediates NF- $\kappa$ B activation, was also measured. In PC-3 cells, IKK $\alpha$  activity was constitutively active, whereas LNCaP cells had minimal IKK $\alpha$  activity that was activated by cytokines. The anti-inflammatory agent ibuprofen inhibited the constitutive activation of NF- $\kappa$ B and IKK $\alpha$  in PC-3 and DU-145 cells, and blocked stimulated activation of NF- $\kappa$ B in LNCaP cells. However, ibuprofen did not directly inhibit I $\kappa$ B- $\alpha$  kinase. The results demonstrate that NF- $\kappa$ B is constitutively activated in the hormone-insensitive prostate tumor cell lines PC-3 and DU-145, but not in the hormone responsive LNCaP cell line. The constitutive activation of NF- $\kappa$ B in prostate tumor cells may increase expression of anti-apoptotic proteins, thereby decreasing the effectiveness of anti-tumor therapy and contributing to the development of the malignant phenotype.

**Keywords:** NF- $\kappa$ B; ibuprofen; prostate; tumor; IKK $\alpha$ ; androgen

The Rel/NF- $\kappa$ B family of transcription factors are activated by a wide range of stimuli, including DNA damage, cytokines and free radicals (Baeuerle and Baltimore, 1996; Miyamoto and Verma, 1995). In unstimulated cells, NF- $\kappa$ B is maintained in an inactive state in the cytoplasm by complexing with members of the I $\kappa$ B inhibitory protein family, including I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$  (Miyamoto and Verma, 1995). The interaction between NF- $\kappa$ B and I $\kappa$ B- $\alpha$  masks NF- $\kappa$ B's nuclear localization signal and inhibits the DNA binding activity of NF- $\kappa$ B (Baeuerle and Baltimore, 1996;

Miyamoto and Verma, 1995). The inducible phosphorylation of serines 32 and 36 of I $\kappa$ B- $\alpha$  (Traencker *et al.*, 1995), by the recently cloned I $\kappa$ B- $\alpha$  kinase, IKK $\alpha$  (Mercurio *et al.*, 1997; Zandi *et al.*, 1998) stimulates the ubiquitination of I $\kappa$ B- $\alpha$ . Ubiquitinated I $\kappa$ B- $\alpha$  is degraded by the 26S proteasome complex (Traencker *et al.*, 1995). NF- $\kappa$ B is then translocated to the nucleus and activates transcription of a variety of genes, including cytokines, cell cycle regulatory proteins, members of the I $\kappa$ B and Rel protein family as well as anti-apoptotic proteins (Baeuerle and Baltimore, 1996; Miyamoto and Verma, 1995; Wang *et al.*, 1998; Wu *et al.*, 1998).

The activation of NF- $\kappa$ B is associated with decreased levels of apoptosis. Cells in which NF- $\kappa$ B activation is inhibited by a dominant negative I $\kappa$ B- $\alpha$  protein exhibit increased levels of apoptosis following exposure to DNA damage or the cytokine TNF $\alpha$  (Tumor Necrosis Factor- $\alpha$ ; Wang *et al.*, 1996). Transgenic mice lacking the p65 sub-unit of NF- $\kappa$ B exhibit increased levels of apoptosis, and fibroblasts from these mice are more sensitive to TNF $\alpha$  induced cell death than normal cells (Beg and Baltimore, 1996). NF- $\kappa$ B can also activate the transcription of genes which suppress apoptosis, through the regulation of caspase activity (Beg and Baltimore, 1996; Wang *et al.*, 1998; Wu *et al.*, 1998). The activation of NF- $\kappa$ B by various agents, including DNA damage, therefore leads to the transcriptional activation of genes that suppress apoptosis (Wang *et al.*, 1996, 1998; Wu *et al.*, 1998). As a consequence of this, inhibition of NF- $\kappa$ B activation leads to increased cell death.

Prostate cancer is the most commonly diagnosed cancer in men. During the early stages of growth, prostate cancer cells are androgen dependent, and tumor growth can be controlled by anti-androgens. However, tumors eventually become unresponsive to anti-androgen therapy and the tumors progress. In this study, we have examined the NF- $\kappa$ B status of a number of well-characterized prostate cancer cell lines that differ in androgen sensitivity. We found that NF- $\kappa$ B levels were constitutively activated in the hormone independent prostate cell lines PC-3 and DU-145, but not in the hormone responsive LNCaP cells. Further, PC-3 cells showed constitutive activation of IKK $\alpha$ , the kinase responsible for phosphorylation of I $\kappa$ B- $\alpha$  and activation of NF- $\kappa$ B. The NSAID (Non-Steroidal Anti-Inflammatory Drug) ibuprofen inhibits the constitutive activation of NF- $\kappa$ B and IKK $\alpha$  in human

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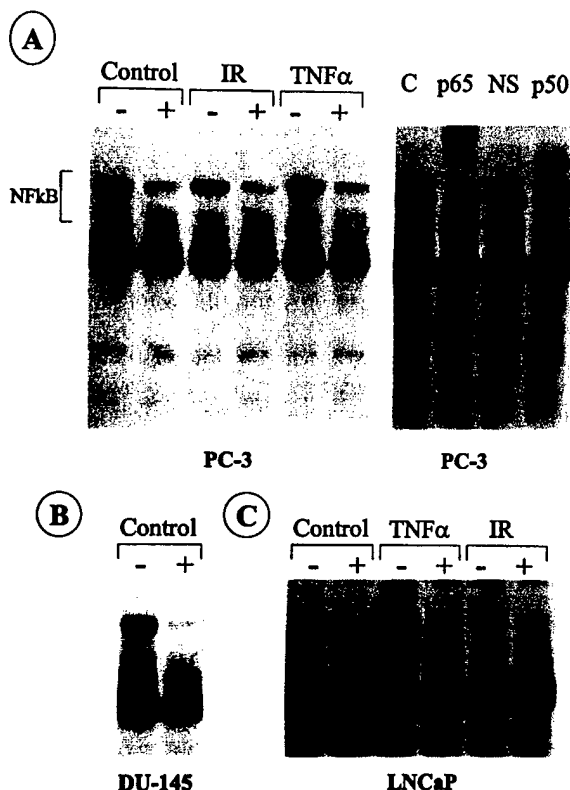
prostate cell lines, although ibuprofen did not directly inhibit IKK $\alpha$ .

The NF- $\kappa$ B complex consists of homo- or heterodimers between Rel family members, including the widely expressed p65/RelA protein (Baeuerle and Baltimore, 1996; Miyamoto and Verma, 1995). Analysis of NF- $\kappa$ B in PC-3 cells by EMSA (Electrophoretic Mobility Shift Assay) revealed multiple DNA-protein complexes (Figure 1a). Antibodies to p65/RelA supershifted the upper, slowly migrating complex. Antibodies to p50 supershifted an additional band below the p65 complex, whereas non-specific

antibodies (NS) were without effect (Figure 1a; right). The faster migrating bands were unaffected by either anti-p65 or p50 antibodies (Figure 1a). The upper, slower migrating bands (indicated by the bracket in Figure 1a) are therefore likely to represent NF- $\kappa$ B complexes, whereas the lower bands represent non-specific complexes. Similar levels of p65 and p50 were observed in DU-145 and LNCaP prostate cancer cells (data not shown). In Figure 1a, untreated PC-3 cells displayed elevated levels of NF- $\kappa$ B DNA binding activity, suggesting that NF- $\kappa$ B was already activated in these cells. This is in contrast to other cell types, where NF- $\kappa$ B is inactive unless exposed to cytokines such as TNF $\alpha$  or to stresses such as DNA damage (Baeuerle and Baltimore, 1996; Basu *et al.*, 1998; Miyamoto and Verma, 1995). When PC-3 cells were exposed to Ionizing Radiation (IR) or TNF $\alpha$ , no further increase in NF- $\kappa$ B DNA-binding activity was seen (Figure 1a). Since PC-3 cells express significant numbers of TNF $\alpha$  receptors (Nakajima *et al.*, 1996), this implies that NF- $\kappa$ B is fully activated in PC-3 cells.

NSAIDs, including ibuprofen, inhibit cyclooxygenases, suppressing prostaglandin production (DeWitt and Smith, 1995). NSAIDs also inhibit the activation of NF- $\kappa$ B (Kopp and Gosh, 1994), are effective suppressors of tumor growth in human colon (DeWitt and Smith, 1995) and can sensitize prostate cells to Ionizing Radiation (Palayoor *et al.*, 1998; Teicher *et al.*, 1996). We used ibuprofen to examine the mechanism of NF- $\kappa$ B activation in prostate tumor cells. When PC-3 cells were exposed to ibuprofen, the levels of constitutive NF- $\kappa$ B binding activity in PC-3 cells was decreased (Figure 1a, +), without affecting the binding of non-specific proteins to be NF- $\kappa$ B consensus oligonucleotide. Ibuprofen also inhibited NF- $\kappa$ B activity in irradiated or TNF $\alpha$  treated PC-3 cells (Figure 1a). Similar results were seen with DU-145 cells, which displayed constitutive activation of NF- $\kappa$ B that was blocked by ibuprofen (Figure 1b). We next examined the NF- $\kappa$ B status of the androgen responsive prostate cell line LNCaP. LNCaP cells had no detectable basal levels of NF- $\kappa$ B, but showed strong activation of NF- $\kappa$ B by both TNF $\alpha$  and Ionizing Radiation (Figure 1c). Ibuprofen inhibited the activation of NF- $\kappa$ B by both TNF $\alpha$  and Ionizing Radiation in LNCaP cells (Figure 1c).

To control for the specificity of ibuprofen towards NF- $\kappa$ B, we also examined the effect of ibuprofen on the DNA-binding activity of the Oct-1 transcription factor. Oct-1 is a ubiquitously expressed transcription factor which binds to the octamer motif (Latchman, 1999). We examined the effect of ibuprofen on the binding of Oct-1 to its consensus recognition site. In Figure 2a, PC-3 cells displayed a single strong DNA binding activity. This binding activity was not competed by excess unlabeled octamer oligonucleotide with a mutation in the octamer binding site (Figure 2a, mutant). Addition of unlabeled octamer oligonucleotide effectively competed for binding (Figure 2a, self). In addition, an Oct-1 antibody supershifted this band whereas a IgG was without effect (Figure 2a, Oct-1 and IgG). This band therefore represents an Oct-1 DNA complex. In both PC-3 and DU-145 cells, Oct-1 binding was not affected by incubation with ibuprofen. LNCaP cells expressed both the Oct-1 protein as well as a second, lower mobility band (Figure 2a).



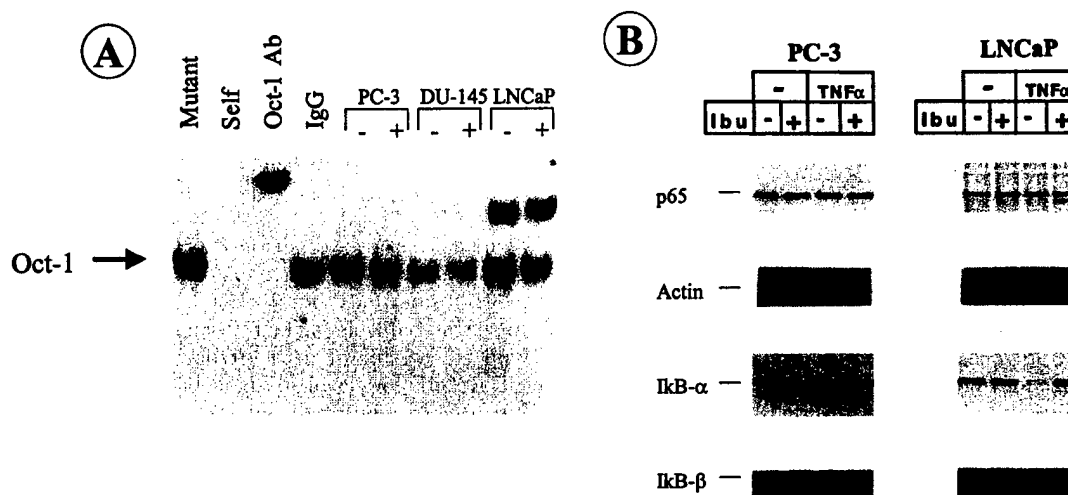
**Figure 1** NF- $\kappa$ B DNA binding activity in PC-3, DU-145 and LNCaP cells. (a) *Left hand panel*: Cells were incubated for 60 min in buffer (–) or 2 mM ibuprofen (+) and then either untreated (Control), Irradiated (IR, 10 Gy) or exposed to TNF $\alpha$  (10 ng/ml). Cell extracts were prepared 1 h later and EMSA carried out as described below. Position of NF- $\kappa$ B complex are indicated. *Right hand panel*: PC-3 cell extracts were incubated for 20 min with buffer (C), antibodies (1  $\mu$ g) to p65, p50 or the transcription factor IRF-1 (NS). NF- $\kappa$ B DNA binding activity was then assessed by EMSA. (b) DU-145 cells were incubated in buffer (–) or ibuprofen (2 mM; +) and EMSA analysis carried. (c) LNCaP cells were incubated for 60 min in buffer (–) or 2 mM ibuprofen (+). Cells were then either untreated (Control), Irradiated (IR) or exposed to TNF $\alpha$  (10 ng/ml) and cell extracts prepared as described below. PC-3, DU-145 and LNCaP cells were maintained as previously described (Palayoor *et al.*, 1998) and treated with ibuprofen in buffer containing HEPES pH 6.8 (20 mM), KCl (120 mM), Glucose (5.5 mM), CaCl $_2$  (1.8 mM) and MgSO $_4$  (1 mM). EMSA. Cell lysates were prepared as described (Basu *et al.*, 1998). EMSA. Reaction contained: Cell lysate (10  $\mu$ g), [ $^{32}$ P]-NF- $\kappa$ B consensus oligonucleotide (AGTTGAGGGG-GACTTCCCCAGGC; 0.5 ng), BSA (20  $\mu$ g), pDI-dC (2  $\mu$ g), Buffer D+ (2  $\mu$ l), Buffer F (4  $\mu$ l) and DTT (1 mM) in 20  $\mu$ l. Buffer D+: (20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP40). Buffer F: (20% FICOLL 400, 100 mM HEPES pH 7.9, 300 mM KCl)

Neither band was affected by the addition of ibuprofen. The identity of the upper band is unknown. The lower band is super-shifted by the Oct-1 antibody, but the upper band is unaffected by the Oct-1 antibody and is not competed by the mutant octamer oligonucleotide. This band may represent either Oct-2 (which also binds the octamer sequence), or a related member of the Octamer binding protein family (Latchman, 1999). We have also seen that ibuprofen does not affect AP-1 binding (unpublished observation), and previous studies demonstrate that ibuprofen activates the DNA binding activity of the Heat Shock Transcription Factor (Soncin and Calderwood, 1996). These observations indicate that ibuprofen exhibits some specificity towards NF- $\kappa$ B, and does not inhibit the DNA binding activity of other transcription factors. Figure 1 therefore demonstrates that PC-3 and DU-145 cells display high basal levels of NF- $\kappa$ B which were not increased by further stimulation, whereas LNCaP cells have low basal NF- $\kappa$ B activity but display rapid activation following stimulation. However, ibuprofen consistently blocked both constitutive and inducible NF- $\kappa$ B activity in all three cell lines.

The activation of NF- $\kappa$ B normally proceeds through the ubiquitination and degradation of the I $\kappa$ B- $\alpha$  inhibitory protein (Baeuerle and Baltimore, 1996; Miyamoto and Verma, 1995). The elevated levels of NF- $\kappa$ B DNA-binding activity seen in PC-3 cells may therefore result from decreased levels of the I $\kappa$ B- $\alpha$  inhibitory protein. To test this hypothesis, PC-3 and LNCaP cell extracts were examined by Western blotting. PC-3 cells contained low levels of I $\kappa$ B- $\alpha$  (Figure 2b), and addition of ibuprofen actually increased the levels of the I $\kappa$ B- $\alpha$  protein (Figure 2b). This is consistent with the decrease in NF- $\kappa$ B DNA binding activity seen in ibuprofen treated PC-3 cells (Figure 1a). TNF $\alpha$  did not alter I $\kappa$ B- $\alpha$  levels in PC-3 cells (Figure 2b). The levels of the I $\kappa$ B- $\beta$  and p65

proteins in PC-3 cells were essentially unchanged by treatment with either TNF $\alpha$  or ibuprofen. Actin levels (measured by Western blotting; Figure 2b) are shown as a loading control. In contrast, exposure of LNCaP cells to ibuprofen did not alter the levels of I $\kappa$ B- $\alpha$ . TNF $\alpha$  decreased the levels of I $\kappa$ B- $\alpha$  protein in LNCaP cells, presumably due to increased degradation of I $\kappa$ B- $\alpha$ , and this correlates with the activation of NF- $\kappa$ B DNA-binding activity seen in Figure 1c. Ibuprofen inhibited TNF $\alpha$  induced I $\kappa$ B- $\alpha$  degradation in LNCaP cells (Figure 2b), and blocked the activation of NF- $\kappa$ B (Figure 1c). Again, neither ibuprofen or TNF $\alpha$  altered the levels of I $\kappa$ B- $\beta$  or p65 protein in LNCaP cells. Figure 2b therefore demonstrates that constitutive activation of NF- $\kappa$ B in PC-3 cells is associated with decreased levels of I $\kappa$ B- $\alpha$  protein. Further, ibuprofen blocks the degradation of I $\kappa$ B- $\alpha$  in both PC-3 and LNCaP cells.

I $\kappa$ B- $\alpha$  levels can be regulated by the inducible phosphorylation of I $\kappa$ B- $\alpha$ , leading to its ubiquitin dependent degradation (Traenckner *et al.*, 1995). IKK $\alpha$  regulates the inducible phosphorylation of I $\kappa$ B- $\alpha$  (Mercurio *et al.*, 1997; Zandi *et al.*, 1998). To determine if IKK $\alpha$  plays a role in the constitutive activation of NF- $\kappa$ B in PC-3 cells, the kinase activity of IKK $\alpha$  immuno-precipitated from PC-3 cells was monitored. PC-3 cells immunoprecipitated with IgG did not phosphorylate an I $\kappa$ B- $\alpha$  fusion protein (Figure 3a). PC-3 cells immunoprecipitated with anti-IKK $\alpha$  antibody phosphorylated I $\kappa$ B- $\alpha$ , but not I $\kappa$ B- $\beta$ . The IKK $\alpha$  antibody therefore specifically immunoprecipitates a kinase activity which phosphorylates I $\kappa$ B- $\alpha$  but not I $\kappa$ B- $\beta$ . The GST fusion tag on the substrates was not phosphorylated by IKK $\alpha$ . In Figure 3a (center), IKK $\alpha$  was immunoprecipitated from PC-3 or LNCaP cells stimulated with TNF $\alpha$ . PC-3 cells exhibited high basal levels of IKK $\alpha$  kinase activity, which was not increased by exposure to TNF $\alpha$  (Figure 3a). In contrast, LNCaP cells had low basal levels of IKK $\alpha$

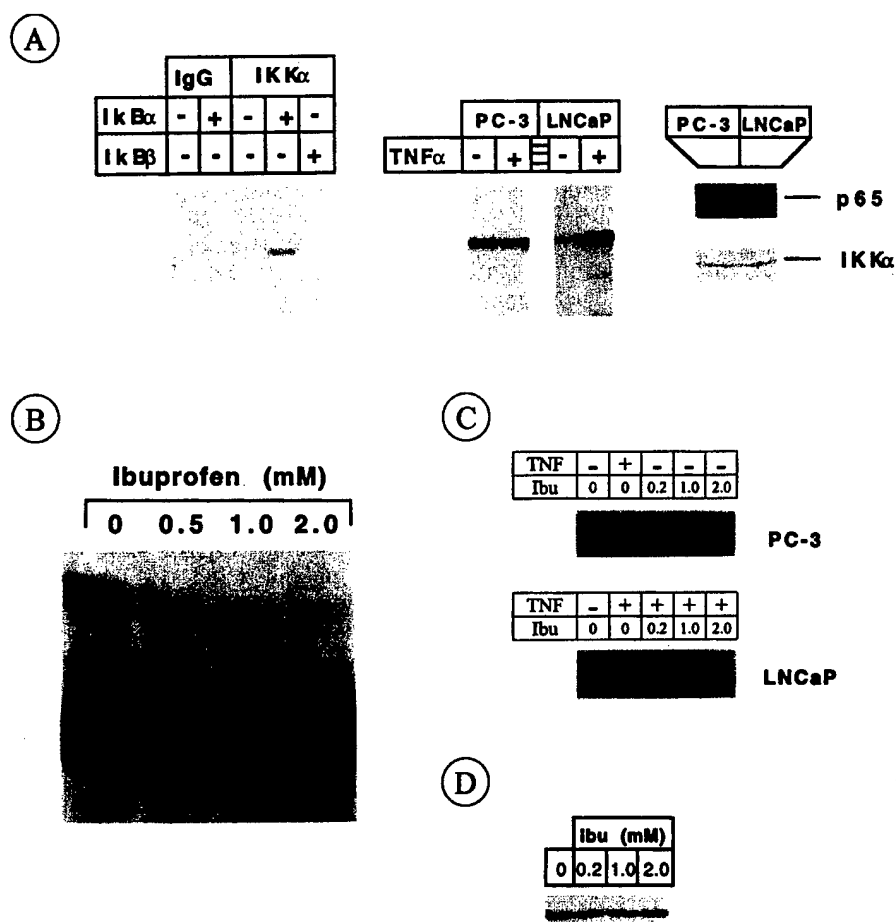


**Figure 2** Effect of ibuprofen on DNA-binding activity of Oct-1. (a) EMSA was carried out using PC-3, DU-145 or LNCaP cell extracts prepared as in Figure 1 and an oligonucleotide corresponding to the octamer binding sequence (TGTCGAATGCAAT-CACTAGAA; 1 ng per reaction). For PC-3 cells, the binding reactions were supplemented with: mutant, 20-fold excess of the mutated octamer oligonucleotide TGTCGAATGCAAGCCACTAGAA; Self, 20-fold excess of unlabeled octamer oligonucleotide; Oct-1 Ab, 1  $\mu$ g of Oct-1 Antibody; NS, 1  $\mu$ g of IgG. Control (-) and ibuprofen (+) treated cells are indicated. (b) PC-3 or LNCaP cells were untreated or exposed to TNF $\alpha$  (10 ng/ml) in the absence (-) or presence (+) of ibuprofen (2 mM). Cell lysates were prepared as in Figure 1 and 18  $\mu$ g of protein examined by Western blotting using antibodies specific for p65, actin, I $\kappa$ B- $\alpha$  or I $\kappa$ B- $\beta$

kinase activity which were stimulated by TNF $\alpha$ . Western blotting of PC-3 and LNCaP cells indicates that both cell lines expressed similar levels of IKK $\alpha$  protein (Figure 2a, right). p65 levels are shown for comparison. The data are consistent with IKK $\alpha$  being constitutively active in PC-3 cells, but requiring stimulation by TNF $\alpha$  for activation in LNCaP cells.

To determine the mechanism by which ibuprofen inhibits NF- $\kappa$ B DNA-binding activity we examined if IKK $\alpha$  was the target for ibuprofen. PC-3 cells were incubated in ibuprofen and NF- $\kappa$ B DNA binding activity (Figure 3b) and IKK $\alpha$  kinase activity measured (Figure 3c). NF- $\kappa$ B DNA binding activity in PC-3 cells was maximally inhibited between 1–2 mM ibuprofen (Figure 3b). Similarly, constitutive

IKK $\alpha$  activity in PC-3 cells was inhibited between 1–2 mM ibuprofen (Figure 3c). In LNCaP cells, TNF $\alpha$  stimulated IKK $\alpha$  activity (Figure 3c), and this activity was also inhibited at 2 mM ibuprofen. Ibuprofen can therefore inhibit the endogenous IKK $\alpha$  kinase activity in both PC-3 and LNCaP cells. To determine if ibuprofen directly inhibits IKK $\alpha$ , IKK $\alpha$  was immunoprecipitated from PC-3 cells. The immunoprecipitated IKK $\alpha$  was then incubated directly with increasing concentrations of ibuprofen prior to the measurement of kinase activity. Under these conditions, we were unable to detect inhibition of IKK $\alpha$  by ibuprofen *in vitro* (Figure 3d). This indicates that ibuprofen may inhibit an upstream regulator of IKK $\alpha$ . Interestingly, low levels of ibuprofen-independent IKK $\alpha$  kinase



**Figure 3** Effect of ibuprofen on the kinase activity of IKK $\alpha$ . (a) *Left*. Immunokinase assays were carried out using PC-3 cell lysates immunoprecipitated with IgG or anti-IKK $\alpha$  antibody and with recombinant I $\kappa$ B- $\alpha$  or I $\kappa$ B- $\beta$  protein as substrate. *Center*. IKK $\alpha$  was immunoprecipitated from untreated or TNF $\alpha$  treated PC-3 or LNCaP cells and IKK $\alpha$  activity assessed using I $\kappa$ B- $\alpha$  as substrate. *Right*. PC-3 or LNCaP cells were examined by Western blotting for p65 or IKK $\alpha$  protein levels. (b) PC-3 cells were exposed to increasing concentrations of ibuprofen for 2 h and the levels of NF- $\kappa$ B DNA binding activity measured by EMSA. (c) PC-3 or LNCaP cells were incubated in the absence (–) or presence (+) of TNF $\alpha$  for 15 min in the presence or absence of the indicated concentration of ibuprofen. IKK $\alpha$  kinase activity was then determined by immunokinase assay. (d) Cell extracts from untreated PC-3 cells were immunoprecipitated with anti-IKK $\alpha$  antibody, washed twice in kinase buffer and then incubated for 15 min in kinase buffer containing the indicated concentrations of ibuprofen. ATP (5  $\mu$ M,  $^{32}$ P-ATP (10  $\mu$ Ci) and I $\kappa$ B- $\alpha$  (0.7  $\mu$ g) were then added and immunokinase activity measured. Kinase assays. Cells were lysed in buffer A (20 mM Tris, pH 7.2; 0.5 M NaCl; 0.5% NP40; 1 mM EDTA; 1 mM DTT; 1 mM PMSF) and the supernatants cleared by centrifugation at 15 kg for 10 min, 1.5  $\mu$ g of IgG or anti-IKK $\alpha$  antibody were prebound to Protein-A/G-Agarose at 4°C for 17 h. Cell extracts (200  $\mu$ g) were incubated with the immobilized antibody for 2 h at 4°C, washed four times in 1 ml of buffer A, and twice in kinase buffer (8 mM MOPS, pH 7.2; 10 mM MgCl<sub>2</sub>; 0.2 mM EDTA). Excess liquid was removed with a hypodermic needle. 30  $\mu$ l of kinase buffer containing ATP (5  $\mu$ M),  $^{32}$ P-ATP (10  $\mu$ Ci) and I $\kappa$ B- $\alpha$  (0.7  $\mu$ g) was added to each tube and allowed to incubate for 10 min at room temperature. Reactions were terminated by the addition of 11  $\mu$ l of 4 $\times$  SDS sample buffer and the phosphorylated proteins separated by SDS-PAGE. GST-I $\kappa$ B- $\alpha$  and GST-I $\kappa$ B- $\beta$  were prepared as previously described by us (Basu et al., 1998)

activity were always detected at doses of ibuprofen of 2 mM (Figure 3c). This may represent the basal IKK $\alpha$  activity in unstimulated cells, and would be unaffected by inhibition of an upstream regulator by ibuprofen.

The results demonstrate that PC-3 and DU-145 prostate cancer cells exhibit constitutive activation of the NF- $\kappa$ B transcription factor, whereas LNCaP cells exhibit TNF $\alpha$ -induced NF- $\kappa$ B activation. Tumor cells derived from breast cancer (Nakshatri *et al.*, 1997) or Hodgkin's disease (Krappmann *et al.*, 1999) also exhibit constitutive activation of NF- $\kappa$ B. In Hodgkin's disease, NF- $\kappa$ B activation is associated with activation of IKK $\alpha$  and decreased levels of I $\kappa$ B- $\alpha$  (Krappmann *et al.*, 1999). Elevated levels of the p65 sub-unit of NF- $\kappa$ B can increase I $\kappa$ B- $\alpha$  levels (Perez *et al.*, 1995) due to transcriptional activation of I $\kappa$ B- $\alpha$  by p65 (Sun *et al.*, 1993). However, the ability of ibuprofen to suppress NF- $\kappa$ B activation is associated with an increase in I $\kappa$ B- $\alpha$  levels and decreased IKK $\alpha$  activity in the absence of any detectable change in p65 levels. We interpret this as an indication that p65 levels themselves do not directly contribute to the activation of NF- $\kappa$ B in PC-3 and DU-145 cells. Instead, the constitutive activation of NF- $\kappa$ B is a consequence of IKK $\alpha$  activation, which in turn phosphorylates I $\kappa$ B- $\alpha$ , triggering its degradation and allowing p65 to accumulate in the nucleus. Our results are the first demonstration that NF- $\kappa$ B is activated in androgen independent prostate tumor cell lines, but not in an androgen-responsive tumor cell line. Analysis of additional prostate tumor cell lines will be needed to determine if NF- $\kappa$ B activation is a common event in androgen independent cells.

The mechanism by which constitutive NF- $\kappa$ B activation occurs is unclear. Both PC-3 and DU-145 cells secrete large amounts of interleukin-6 and other cytokines (Nakajima *et al.*, 1996), whereas LNCaP cells secrete much lower levels. Cytokines can activate NF- $\kappa$ B, and NF- $\kappa$ B can itself activate the transcription of cytokines such as interleukin-6 (Baeuerle and Baltimore, 1996). Chronic autocrine stimulation of the NF- $\kappa$ B may therefore account for constitutive NF- $\kappa$ B activation in PC-3 and DU-145 cells. However, whether the high levels of interleukin-6 (or other factors) secreted by PC-3 and DU-145 cells are the cause or consequence of NF- $\kappa$ B activation is not known. An alternative mechanism for constitutive activation of NF- $\kappa$ B in PC-3 cells is activation of an internal signal transduction pathway, perhaps due to mutation or inappropriate expression of regulatory proteins in these tumor cells. For example, overexpression of the anti-apoptotic protein bcl-2 protein can activate NF- $\kappa$ B and suppress apoptosis (de Moissac *et al.*, 1998), and many advanced prostate tumors express bcl-2 (Apakama *et al.*, 1996). The constitutive activation of NF- $\kappa$ B in prostate tumor cells may have a number of consequences. These include increased production of NF- $\kappa$ B regulated cytokines as well as suppression of the apoptotic response. NF- $\kappa$ B can repress transcription of the Androgen Receptor (Supakar *et al.*, 1995) and can bind to and inhibit Androgen Receptor function (Palvimo *et al.*, 1996). In addition, neither PC-3 nor DU-145 cells express detectable levels of the Androgen Receptor, whereas LNCaP cells contain high levels of the receptor (Tilley *et al.*, 1990). This suggests that

constitutive activation of NF- $\kappa$ B in PC-3 and DU-145 cells may also inhibit expression of the Androgen Receptor. The activation of NF- $\kappa$ B may therefore contribute to the emergence of androgen-independent prostate tumor cells and the development of the malignant phenotype.

IKK $\alpha$  was inhibited in ibuprofen treated cells but this was not due to direct inhibition of IKK $\alpha$  by ibuprofen. This implies that ibuprofen inhibits an upstream regulator of IKK $\alpha$ . Ibuprofen also inhibited the activation of NF- $\kappa$ B by both TNF $\alpha$  and DNA damage (Figure 1c). This suggests that ibuprofen inhibits a signaling component which is common to the pathways utilized by TNF $\alpha$  and Ionizing Radiation to activate NF- $\kappa$ B. Several members of the MEKK kinase family have been implicated in the upregulation of the IKK $\alpha$  enzyme complex, including NIK (Ling *et al.*, 1998) and MEKK1 (Lee *et al.*, 1998). IKK $\alpha$  is part of a large multiprotein complex including IKK $\alpha$ , IKK $\beta$ , NIK, I $\kappa$ B- $\alpha$  and - $\beta$ , NF- $\kappa$ B sub-units as well as other unidentified components (Cohen *et al.*, 1998). Ibuprofen may be an inhibitor of the upstream regulator of this complex, or a generic inhibitor of all MEKK kinases. Other NSAIDs, such as aspirin, can inhibit IKK $\beta$ , the kinase which phosphorylates I $\kappa$ B- $\beta$  (Yin *et al.*, 1998). However, we have been unable to detect any effect of ibuprofen on I $\kappa$ B- $\beta$  protein levels (Figure 2b), although it is possible that ibuprofen may also inhibit IKK $\beta$ .

Ibuprofen alone does not affect DU-145 or LNCaP tumor growth in mice (Teicher *et al.*, 1996). In culture, 1 mM ibuprofen does not cause significant growth delay of PC-3 or DU-145 cells, although higher doses (2 mM and above) can cause growth delay and increase the apoptotic rate (Palayoor *et al.*, 1998). However, ibuprofen can sensitize DU-145, PC-3 and LNCaP cells to radiation both in culture and in animal tumor models (Palayoor *et al.*, 1998; Teicher *et al.*, 1996). Ibuprofen therefore only enhances cell death in combination with an associated genotoxic event. The serum levels of ibuprofen achieved clinically are of the order of 0.2 mM (Laska *et al.*, 1986), although higher levels can be tolerated acutely. The inhibition of IKK $\alpha$  seen here *in vitro* at 1 mM ibuprofen (Figure 3c) may therefore be achievable *in vivo*.

NSAIDs inhibit COX-1 and COX-2, the enzymes responsible for the synthesis of prostaglandins. NSAIDs are also effective suppressors of tumor growth in human colon (DeWitt and Smith, 1995). However, the concentrations of NSAIDs required to inhibit prostaglandin synthesis are much lower than those required to exert anti-tumor effects. Whether NSAIDs suppress tumor growth through inhibition of prostaglandin synthesis is therefore unclear. NF- $\kappa$ B can also activate the transcription of a variety of anti-apoptotic genes, including TRAF1 and -2, cIAP-1 and -2 and IEX-1L, leading to suppression of apoptosis, perhaps by modulation of caspase activity (Wang *et al.*, 1998; Wu *et al.*, 1998). Inhibition of NF- $\kappa$ B by genetic methods is associated with increased apoptotic cell death following exposure to DNA damage or to TNF $\alpha$  (Beg and Baltimore, 1996; Wang *et al.*, 1996). In addition, we have shown that ibuprofen sensitizes prostate tumor cells PC-3 and DU-145 to radiation therapy in both tissue culture and animal models (Palayoor *et al.*, 1998; Teicher *et al.*, 1996). The

constitutive activation of NF- $\kappa$ B in prostate tumor cells may result in the expression of high levels of anti-apoptotic proteins. This, in turn, may suppress the normal apoptotic response and allow the cells to survive DNA damage, decreasing the effectiveness of anti-tumor therapy. The inhibition of NF- $\kappa$ B activation in these prostate tumor cells by ibuprofen may therefore contribute to increased cell death through inhibition of transcription of anti-apoptotic genes. NSAIDs may prove to be valuable drugs for use in the treatment of tumors, and may provide a starting

point for the rational design of agents which specifically inhibit the activation of NF- $\kappa$ B. Further, the ability of NSAIDs to act as suppressor of tumor growth may be, in part, related to their ability to inactivate NF- $\kappa$ B.

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